## REMARKS

In the Final Action dated March 9, 2005, claims 40, 42-47 and 57-58 are pending and under consideration. Claims 40, 42-47 and 57-58 have been rejected under 35 U.S.C. §101 as allegedly not supported by either a specific and substantial asserted utility or a well established utility. Claims 40, 42-47, 57 and 58 have also been rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enabling support.

This Response addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance or at least in better condition for appeal. Favorable consideration of all pending claims is therefore respectfully requested.

In the first instance, by way of the instant amendment, Applicants have added claim 59. Claim 59 is a further delineation of claim 57. Support for claim 59 can also be found in the specification, e.g., Table 3 starting at page 29 and Example 8 on page 43. No new matter is introduced. In an effort to favorably advance the prosecution, Applicants have also canceled claims 46-47, without prejudice. Applicants reserve the right to file a continuation application directed to the subject matter of claims 46-47.

The Examiner rejects Claims 40, 42-47 and 57-58 under 35 U.S.C. §101 as allegedly not supported by either a specific and substantial asserted utility or a well established utility. The Examiner acknowledges that Applicants, in response to the previous Official Action dated June 1, 2004, argued that the claimed receptor "NR6" is explicitly characterized in the specification as a member of the haemopoietin receptor family. The Examiner acknowledges that Applicants also argued that Dr. Hilton's Declaration previously submitted in the present case discloses that lack of NR6 results in reduced blood cell production and that the fact that NR6 is

lethal during embryonic development or immediately after birth enables the detection of potential birth defects or potential dysfunction to haemopoiesis.

In the present Action, the Examiner explicitly states that "[i]t is found persuasive that the NR6 used to establish the knockout mice would have a 'real world' utility for diagnostic purposes, because it can be used in predicting birth defects. It is also found persuasive that the NR6 receptor which results in reduced blood cell production, would also be found useful therapeutically, in the regulation of haemopoieses." See second paragraph on page 3 of the Official Action. However, the Examiner alleges that the present specification does not disclose which of the disclosed NR6 polypeptides was actually used to carry out the knockout mice experiments or was shown to reduce blood cell production.

The Examiner asserts that Claims 40, 42-47 and 57-58 are not supported by either a specific and substantial asserted utility or a well established utility unless Applicants disclose which one of the polypeptides of SEQ ID NOS: 13, 17, 15, 25 or 44 was found to result in reduced blood cell production and was found lethal during embryonic development or immediately after birth. Notably, the Examiner states that once Applicants disclose the above-mentioned polypeptide, the rejection under 35 U.S.C. § 101 will be withdrawn for that polypeptide. In asserting that Claims 40, 42-47 and 57-58 are not supported by either a specific and substantial asserted utility or a well established utility, the Examiner alleges that SEQ ID NOs: 13, 17, 15, 25 or 44 are disparate sequences. See middle of page 3 of the Official Action.

Applicants observe that SEQ ID NOs: 13, 15 and 17 represent the amino acid sequence of mNR6.1, amino acid sequence of mNR6.2 and a partial amino acid sequence of mNR6.3, respectively. See Table 3 on page 29 of the specification. Applicants also observe that SEQ ID NO: 25 represents the amino acid sequence of human clone HFK-66 and SEQ ID NO: 44 represents the amino acid sequence of human NR6. See Table 3.

In response, Applicants respectfully submit that the NR6 polypeptides described in the specification are not disparate polypeptides but are alternatively spliced forms or species homologs expressed by a single NR6 gene. Applicants respectfully direct the Examiner's attention to the specification on pages 30-31 and Table 3 on page 29 where the specification discloses that SEQ ID NOs: 13, 15 and 17 represent translation products of three alternatively spliced transcripts of a single gene (i.e., the murine NR6 gene). Applicants also submit that as described in Example 9 on page 44, the genomic structure of the human homolog was identified as very similar to that of murine NR6. Applicants submit that a single full-length human NR6 cDNA clone was isolated and sequenced as the polypeptide of SEQ ID NO: 44, which is the human homolog of the murine NR6 polypeptide mNR6.2 as set forth in SEQ ID NO: 15.

Applicants respectfully submit that the specification discloses that an NR6 knockout mice recognized by the present invention provides a useful model for diagnosing developmental defects. See the specification on page 32, lines 7-10. Since the production of the NR6 knockout mouse involved removal of most of the NR6 gene (see Figure 5), Applicants respectfully submit that each of the murine NR6 splicing forms mNR6.1, mNR6.2 and mNR6.3 (as set forth in SEQ ID NOs: 13, 15 and 17, respectively) are affected and not produced in the knockout mouse.

Thus, Applicants respectfully submit that, contrary to the Examiner's allegation, at least three polypeptide sequences disclosed in the specification were affected in the knockout mice experiment. Applicants also submit that the phenotype of the knockout mice demonstrate that the NR6 gene and its various expression products are important in development and haemopoiesis, the lack of which can implicate developmental defects.

Additionally, Applicants respectfully submit that given the high level of structural similarity between the human NR6 and the murine NR6, it is expected that the human NR6

would also be functionally active. In this regard, Applicants refer to Knappskog et al. (*Am. J. Hum. Genet.* 72: 375-383, 2003) (copy enclosed as Exhibit A) who describe that a mutation in Exon 5 of the human NR6 gene is responsible for cold-induced sweating syndrome in subjects homozygous for the mutation.[1] Exhibit A provides the results of a mapping study and shows that screening for mutations in the NR6 (aka CRLF1) gene provides a useful diagnostic marker for the cold-inducing sweating syndrome.

Moreover, Applicants respectfully submit that both the murine and human NR6 forms are suitable for use in diagnosis or for use prognostically in prediction of birth defects. For example, when the NR6 gene is knocked out (in mice) or mutated (in humans), a suckling defect is observed in the homozygous knockout or homozygous mutant form, respectively. In this regard, Applicants refer to an article co-authored by some of the present inventors (Alexander et al., *Current Biology* 9 (11) 605-608, 1999) (copy enclosed as Exhibit B). Exhibit B discloses the suckling defect and reduced level of haemopoietic progenerator cells in homozygous knockout mice.[2] Applicants also submit that like NR6 homozygous knockout mice, it has been reported that human subjects with NR6 expression failed to suckle spontaneously, which indicates that the mouse and human NR6 forms are functional, as well as structural, homologs.[3]

Finally, Applicants respectfully submit that 35 U.S.C. §101 only requires at least one utility in a patent application.

In view of the foregoing, it is respectfully submitted that the claimed receptor is supported by a specific and substantial asserted utility. Therefore, the rejection of Claims 40, 42-

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[1] NR6 is referred to in Exhibit A as CRLF1.
[2] NR6.1 described in the Exhibit B (see Figure 1) is NR6.2 of the present application, and NR6.2 in the Exhibit B is NR6.1 of the present application.
[3] No spliced variants of human NR6 have been isolated or reported.

47, 57 and 58 under 35 U.S.C. § 101 is overcome and withdrawal thereof is respectfully requested.

Claims 40, 42-47, 57 and 58 are also rejected under 35 U.S.C. §112, first paragraph. Specifically, the Examiner alleges that since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

Applicants respectfully submit that, as discussed above, the claimed invention is supported by a specific and substantial asserted utility. Thus, one skilled in the art would know how to make and use the claimed invention, without undue experimentation. As such, the rejection of claims 40, 42-47, 57 and 58 under 35 U.S.C. §112, 1st paragraph, is overcome and withdrawal therefore is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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# Cold-Induced Sweating Syndrome Is Caused by Mutations in the *CRLF1* Gene

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**In 1978, Sohar et al. described a strikingly peculiar syndrome in two Israeli sisters. These young women responded to environmental temperatures of 18°C–7°C with profuse sweating on large segments on their back and chest. Both had additional abnormalities, including a high-arched palate, nasal voice, depressed nasal bridge, inability to fully extend their elbows, and kyphoscoliosis. We have observed this disorder in two Norwegian brothers. Genomewide screening in the two families, followed by saturation marker studies and linkage analysis, identified a 1.4-Mb homozygous candidate region on chromosome 19p12. The maximum multipoint LOD score was 4.22. In both families, DNA sequencing of 25 genes within the candidate region identified potentially deleterious *CRLF1* sequence variants that were not found in unaffected control individuals. Our findings confirm that the cold-induced sweating syndrome is an autosomal recessive disorder that is probably caused by impaired function of the *CRLF1* gene, and they suggest important developmental functions for human CRLF1.**

## Introduction

The cold-induced sweating syndrome (CISS [MIM 272430]) was first described by Sohar et al. (1978). Two Israeli sisters experienced profuse sweating, induced by cool surroundings, on large segments of their back and chest. They also had some additional abnormalities, including a high-arched palate, nasal voice, depressed nasal bridge, inability to fully extend their elbows, and kyphoscoliosis. Their parents shared a common grandfather, suggesting that the observed condition represented a novel syndrome inherited as an autosomal recessive trait. Since this initial description, no confirming case of CISS with reference to the original publication has been described. Thus, the reported disorder is probably very rare.

We have observed a clinical phenotype in two Norwegian brothers that is similar to the one described in the Israeli sisters. No parental consanguinity was known, but genealogical studies revealed several shared ancestors, the closest of which was found nine generations back. Thus, also in the affected Norwegian brothers, homozygosity for a mutant gene inherited from a common ancestor constituted a likely mechanism for this disorder. Exploiting this unique situation, we employed a combination of coarse-scale homozygosity mapping, based on the Israeli inbred sibship with a common great-grandfather, and finer-scale localization, based on the Norwegian sibship with distant common ancestors. Thus, on the basis of only four patients, we have identified the candidate chromosomal segment, the candidate gene, and the likely causative mutations.

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## Material and Methods

### *Genotyping*

Genomic DNA was isolated from whole blood by using an ABI 341 Nucleic Acid Extractor (PE Applied Biosystems). A genomewide scan was performed using a set of 400 microsatellite markers with an average spacing of 10 cM (ABI Prism Linkage Mapping Set MD, version 2). PCR and pipetting were performed using the ABI Catalyst 800 Turbo Lab station. The PCR products were analyzed using an ABI 310 Genetic Analyzer and the Genescan Analysis software (PE Applied Biosystems). High-density mapping was performed by employing markers identified in various databases (see the GenLink, Cooperative Human Linkage Center, Entrez Genome, Genome Database, and Center for Medical Genetics, Marshfield Medical Research Foundation, Web sites). On the basis of chromosome 19 draft sequences from Lawrence Livermore National Laboratories (see the LLNL Human Genome Center Web site), anonymous repeated (CA) sequences were identified, primers were constructed, and amplified fragments were probed for variants in the Norwegian nuclear family. The PCR primer sequences and population heterozygosity frequencies of these markers are given in table A (online only).



### *Linkage Analysis*

Two-point and multipoint linkage analyses were performed using the Mlink and Linkmap programs of the Fastlink software package (Cottingham et al. 1993). Initially, single-point LOD scores were used to identify all regions consistent with a recessive pattern of inheritance in both families. Those regions were then subjected to fine mapping with additional genetic markers. After identification of the candidate region on chromosome 19p12, multipoint analysis (limited to three markers by running time of the software) was performed, to determine the statistical significance of the finding. We used a disease-allele frequency of 0.001 and penetrances of 0 for carriers and noncarriers and 0.99 for homozygous affected individuals. In the initial genome scan, equal allele frequencies were assumed, but, for the fine mapping in the Norwegian family, allele frequencies were determined in 50 unaffected Norwegian control individuals. We also used a method based on the theoretical work of Durham and Feingold (1997), to estimate the probability that the homozygosity observed in the Norwegian pedigree is a false positive (i.e., a chance occurrence, not caused by linkage to the disease locus). We applied equations 1 and 4 of Durham and Feingold (1997) and considered all inbreeding loops of different lengths to be independent, resulting in a conservative $P$ value.

### *DNA Sequencing and Mutation Detection*

PCR primers for amplification of exons and flanking intron sequences in the 1.4-Mb region were designed using the Oligo 6.3 software (Molecular Biology Insights). PCR amplification was performed under standard conditions, using AmpliTaq Gold (PE Applied Biosystems) or *Taq* polymerase (Qiagen). After amplification, the PCR products were treated with SAP/exonuclease I (Amersham), were sequenced using the ABI Prism BigDye terminator and sequencing kit version 2, and were analyzed on an ABI 3100 Genetic Analyzer (PE Applied Biosystems). A list of our SNP findings is given in table B (online only). Sequencing primers are available on request. DNA sequences were analyzed using the Staden software package (Bonfield et al. 1998).

### *Control Samples*

DNA samples were obtained from 200 ostensibly healthy local Norwegian blood donors and 50 Israeli matched control individuals of an ethnic background similar to the patients.

### *Screening Tests for the* CRLF1 *Sequence Variants*

The c.844_845delGT mutation was verified by PCR amplification by forward (5′-GCAGAGGGAAGAGGAGGAAAACAGA-3′) and reverse (5′-CACACCACTATGCGACAGAATGAG-3′) primers of exon 5 of the *CRLF1* gene, followed by analysis of the fluorescence-labeled PCR product on an ABI 310 Genetic Analyzer. The R81H mutation destroys a natural *Hha*I restriction site in exon 2 of the *CRLF1* gene, and this formed the basis for a rapid screening for this mutation. Exon 2 of the *CRLF1* gene was PCR amplified using the exon 2 forward (5′-ATTTAACCCAACTGATCTCTACCTT-3′) and reverse (5′-TGAAAGACCTGCATAGCCAT-3′) primers, followed by digestion by *Hha*I and separation on an 3% Nusieve agarose gel (FMC). *Hha*I digestion of the PCR-amplified products from normal chromosomes gives two bands (286 and 290 bp), whereas no cutting of the PCR product amplified from mutant chromosomes was observed. We found no simple way of detecting L374R, and a search for this variant was performed by sequencing the proper exon.

### *Clinical Findings*

A brief clinical description of the Israeli sisters has been given elsewhere (Sohar et al. 1978). Both sisters noted the cold-induced sweating at age 16–17 years, shortly after menarche. Their problem has now persisted unchanged for 25 years. The sweating reaction to cold exposure always starts at the same point, in the presternal region in one and in the left hand in the other. It quickly spreads to the rest of the affected areas, distributed as patches, above the waist, on the chest and back. In the affected areas, no sweating occurs at warm temperatures or during febrile episodes. No medical treatment or remedy has so far been helpful in relieving this socially embarrassing disorder. Renewed x-ray examinations show that both sisters have thoracolumbar scoliosis, moderate (30°–35°) in the older sister and less pronounced in the younger sister. Neither of these patients had feeding difficulties in the newborn period. Both sisters have four children, none of whom are affected with this disorder.

The Norwegian brothers were born at term, after uneventful pregnancies. The older would not suckle in the neonatal period and was admitted, dehydrated, to the hospital at 5 d old. He was fed first by a nasogastric tube and subsequently by a special sucking device intended for newborn lambs. Because of continued severe feeding problems, complicated by bronchopulmonary and urinary tract infections, he was treated in the neonatal ward for 3 mo. His younger brother was admitted at 1 d old, primarily because of respiratory problems. Also, this newborn baby did not suckle spontaneously and had to be fed in ways similar to those used for his older brother. Both have problems

with fully opening their mouths, rendering ordinary dental work difficult. While playing in the snow, the older brother has repeatedly experienced frostbite in his hands, which was, on one occasion, severe, requiring professional treatment. Likewise, he can hold his palms in a flame or put his hands in boiling water without any sensory pain.

Both of these Norwegian boys have severe progressive kyphoscoliosis. In the younger brother, an S-shaped scoliosis rapidly progressed over a period of 6 mo, at age 13 years. At that time, the major curve measured 47°, and the kyphosis measured 70° (Cobb angle [Cobb 1948]). Posterior-spine surgery was performed, the deformity was corrected, and thoracic vertebrae 3–11 were fused. However, with time, severe kyphosis developed above the fused part. The older brother was first seen by an orthopedic surgeon at age 18 years. He then had both a severe kyphosis (Cobb angle 90°) and scoliosis that was somewhat less pronounced. Also, this patient underwent spine surgery, but a combined anterior and posterior approach was chosen because of severe stiffness. Posterior instrumentation was done, and fusion between thoracic vertebrae 2 and 12 was achieved with satisfactory correction.

The procedures performed on these brothers are considered to be very painful in the postoperative period. However, it was noted that the boys had unusually low demand for pain-relieving medication and seemingly were not bothered by the postoperative pain. During surgery on the older brother, the surgeon noted unusually lightly colored muscle ("like chicken meat"). A biopsy was performed, and the finding was described as "muscular atrophy."

Both brothers have short hands with pronounced clinodactyly and tapering of fingers. They cannot fully extend their elbows (30° deficit). Also, their toes are somewhat short, and both have flat feet. They have insufficient activity of facial muscles, leading to expressionless faces; instead of a smile, a grin results. Their sweating problem was noted at ~7 years of age. The patchwise distribution of affected areas much resembles those described in the Israeli sisters. These areas do not sweat at warm temperatures, during fever episodes, or during exercise. The mother sometimes had to cool her overheated children by putting their feet in cold water. Subtropical environment does not bother these patients. They can stay in bright sunlight without feeling the heat and have no desire to take their clothes off for cooling.

### *Genealogical Studies*

Until the 20th century, the majority of the Norwegian population was attached to a single locality throughout life. A cumulative population-inbreeding coefficient has been estimated at 0.0027 (Gedde-Dahl 1973). Many rural communities in Norway have produced printed local histories, often including volumes of painstakingly gathered genealogical data for each farm, back to the first church records and censuses (17th century). For communities without such printed sources, online data from national censuses and church records were used (see the Digitalarkivet Web site). These tools made it possible to identify, by name, all of the Norwegian brothers' 32 ancestors, five generations back, all of whom were born in the first half of the 19th century. All ancestral lines were pursued as far back as possible. Of the 16 ancestors in each line, 3 in the paternal line and 8 in the maternal line originated from the same rural community. It was possible to trace the majority of these ancestral branches back to the 17th century, and it was possible to trace some branches even further back. The first common ancestor was identified nine generations back (fig. 1*A*, indicated by the arrow). Another five, six, three, two, and one new common ancestral couples were identified 10, 11, 12, 13, and 14 generations back, respectively. Nearly 1,000 ancestors were identified by name, and a very complex pedigree emerged.

## Results

### *Linkage Analysis*

When genomewide screening was performed, using markers with an average spacing of 10 cM, three possible candidate regions could not readily be excluded. Ambiguous results owing to limited heterozygosity were obtained in regions on chromosomes 3q and 21. The results of a total of 12 additional markers, distributed between the screening markers, excluded these regions as true candidates. However, on chromosome 19, the Israeli sisters and the Norwegian brothers had inherited, from their parents, a pair of common segments spanning <60 Mb and <48 Mb, respectively (table 1). Within the 42-Mb overlapping candidate segment, the Israeli sisters were homozygous for only two nonadjacent markers, D19S221 and D19S414. Homozygosity was not observed for any of the initial screening markers in this region (D19S221-D19S414) in the Norwegian sibship. Detailed mapping of this region showed that the two Israeli sisters were homozygous for a segment, encompassing 28.8 Mb, that was not revealed by the screening markers (fig. 1 and table 1). Markers D19S221 and D19S414 were both positioned outside this segment. Subsequently, saturation marker studies, as well as SNPs detected by DNA sequencing, demonstrated a 1.4-Mb region of homozygosity within the candidate segment in the Norwegian brothers (fig. 1 and table 1).

We performed multipoint linkage analysis, to determine

**Table 1**

**Genotypes of Chromosome 19 Markers**

| Marker[a] | Genotype[b] in Pedigree | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | X-1 | | X-2 | | XI-1 | | XI-2 | | III-2 | | IV-1 | | IV-2 | | IV-3 | | IV-4 | | IV-5 | |
| D19S209 | 251 | 251 | 247 | 243 | 251 | 243 | 251 | 247 | 246 | 250 | 248 | 250 | 248 | 250 | 244 | 246 | 248 | 250 | 244 | 250 |
| D19S216 | 265 | 269 | 271 | 267 | 265 | 271 | 265 | 271 | 259 | 265 | 265 | 265 | 267 | 265 | 265 | 259 | 267 | 265 | 265 | 265 |
| D19S884 | 107 | 97 | 107 | 107 | 107 | 107 | 107 | 107 | 103 | 107 | 91 | 107 | 97 | 107 | 97 | 103 | 97 | 107 | 91 | 107 |
| D19S221 | 88 | 104 | 104 | 106 | 88 | 104 | 88 | 104 | 98 | 98 | 93 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 93 | 98 |
| D19S226 | 254 | 254 | 246 | 250 | 254 | 246 | 254 | 246 | 240 | 254 | 254 | 254 | 238 | 254 | 238 | 240 | 238 | 240 | 254 | 254 |
| D19S929 | 251 | 253 | 251 | 253 | 251 | 251 | 251 | 251 | 253 | 249 | 251 | 249 | 249 | 249 | 249 | 253 | 249 | 253 | 251 | 249 |
| D19S841 | 245 | 245 | 235 | 235 | 245 | 235 | 245 | 235 | 247 | 243 | 235 | 243 | 243 | 243 | 243 | 247 | 243 | 247 | 235 | 243 |
| D19S588 | 162 | 148 | 170 | 170 | 162 | 170 | 162 | 170 | 149 | 149 | 149 | 149 | 166 | 149 | 166 | 149 | 166 | 149 | 149 | 149 |
| D19S244 | 138 | 109 | 126 | 99 | 138 | 126 | 138 | 126 | 101 | 105 | 105 | 105 | 90 | 149 | 90 | 101 | 90 | 101 | 105 | 105 |
| D19S930 | 181 | 196 | 183 | 181 | 181 | 183 | 181 | 183 | 187 | 183 | 198 | 183 | 189 | 149 | 189 | 187 | 189 | 187 | 198 | 183 |
| D19S899 | 102 | 109 | 104 | 112 | 102 | 104 | 102 | 104 | 103 | 105 | 101 | 105 | 107 | 149 | 107 | 103 | 107 | 103 | 101 | 105 |
| D19S410 | 155 | 168 | 173 | 155 | 155 | 173 | 155 | 173 | 162 | 154 | 154 | 154 | 171 | 149 | 171 | 162 | 171 | 162 | 154 | 154 |
| D19S579 | 163 | 179 | 179 | 171 | 163 | 179 | 163 | 179 | 175 | 175 | 175 | 175 | 171 | 149 | 171 | 175 | 171 | 175 | 175 | 175 |
| D19S429 | 231 | 235 | 239 | 231 | 235 | 239 | 235 | 239 | 231 | 231 | 231 | 231 | 235 | 149 | 235 | 231 | 235 | 231 | 231 | 231 |
| D19S915 | 109 | 101 | 107 | 111 | 109 | 107 | 109 | 107 | 89 | 109 | 89 | 109 | 107 | 149 | 107 | 89 | 107 | 89 | 89 | 109 |
| D19S1037 | 119 | 119 | 123 | 123 | 119 | 123 | 119 | 123 | 115 | 131 | 135 | 131 | 115 | 149 | 115 | 115 | 115 | 115 | 135 | 131 |
| D19S212 | 193 | 197 | 193 | 205 | 193 | 193 | 193 | 193 | 197 | 205 | 193 | 205 | 197 | 149 | 197 | 197 | 197 | 197 | 193 | 205 |
| D19S460 | 128 | 130 | 128 | 122 | 130 | 128 | 130 | 128 | 128 | 122 | 130 | 122 | 128 | 149 | 128 | 128 | 128 | 128 | 130 | 122 |
| D19S898 | 174 | 184 | 190 | 180 | 174 | 190 | 174 | 190 | 190 | 179 | 177 | 179 | 190 | 149 | 190 | 190 | 190 | 190 | 177 | 179 |
| M6A | 196 | 212 | 202 | 198 | 196 | 202 | 196 | 202 | | | | | | | | | | | | |
| M5A | 187 | 117 | 201 | 203 | 187 | 201 | 187 | 201 | | | | | | | | | | | | |
| M1A | 245 | 247 | 245 | 237 | 245 | 245 | 245 | 245 | | | | | | | | | | | | |
| M3A | 228 | 230 | 228 | 220 | 228 | 228 | 228 | 228 | | | | | | | | | | | | |
| D19S895 | 127 | 122 | 127 | 127 | 127 | 127 | 127 | 127 | 131 | 123 | 133 | 123 | 131 | 123 | 131 | 131 | 131 | 131 | 133 | 123 |
| M4A | 170 | 177 | 170 | 170 | 170 | 170 | 170 | 170 | | | | | | | | | | | | |
| D19S566 | 154 | 160 | 154 | 156 | 154 | 154 | 154 | 154 | 154 | 142 | 156 | 142 | 154 | 142 | 154 | 154 | 154 | 154 | 156 | 142 |
| D19S443 | 128 | 128 | 128 | 128 | 128 | 128 | 128 | 128 | 126 | 126 | 126 | 126 | 126 | 126 | 126 | 126 | 126 | 126 | 126 | 126 |
| D19S603 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 | 136 |
| M1 | 185 | 185 | 185 | 185 | 185 | 185 | 185 | 185 | | | | | | | | | | | | |
| M2 | 156 | 158 | 156 | 158 | 156 | 156 | 156 | 156 | | | | | | | | | | | | |
| M3 | 163 | 165 | 163 | 165 | 163 | 163 | 163 | 163 | | | | | | | | | | | | |
| M5 | 201 | 189 | 201 | 201 | 201 | 201 | 201 | 201 | | | | | | | | | | | | |
| M4 | 170 | 177 | 170 | 170 | 170 | 170 | 170 | 170 | | | | | | | | | | | | |
| M6 | 188 | 186 | 186 | 184 | 188 | 186 | 188 | 186 | | | | | | | | | | | | |
| D19S546 | 314 | 314 | 314 | 314 | 314 | 314 | 314 | 314 | 314 | 284 | 314 | 284 | 314 | 284 | 314 | 314 | 314 | 314 | 314 | 284 |
| D19S407 | 209 | 209 | 216 | 216 | 209 | 216 | 209 | 216 | 202 | 210 | 210 | 210 | 202 | 210 | 202 | 202 | 202 | 202 | 210 | 210 |
| M8 | 155 | 148 | 153 | 148 | 155 | 153 | 155 | 153 | | | | | | | | | | | | |
| D19S911 | 231 | 231 | 225 | 231 | 231 | 225 | 231 | 231 | 231 | 227 | 229 | 227 | 231 | 227 | 231 | 231 | 231 | 231 | 229 | 227 |
| D19S602 | 103 | 105 | 109 | 103 | 103 | 109 | 103 | 103 | 109 | 103 | 115 | 103 | 109 | 103 | 109 | 109 | 109 | 109 | 115 | 103 |
| D19S925 | 262 | 266 | 266 | 268 | 262 | 266 | 262 | 268 | 266 | 262 | 266 | 262 | 266 | 262 | 266 | 266 | 266 | 266 | 266 | 262 |
| D19S215 | 259 | 243 | 249 | 257 | 259 | 249 | 259 | 257 | 243 | 257 | 249 | 257 | 243 | 257 | 243 | 243 | 243 | 243 | 249 | 257 |
| D19S910 | 239 | 251 | 239 | 239 | 239 | 239 | 239 | 239 | 239 | 239 | 237 | 239 | 239 | 239 | 239 | 239 | 239 | 239 | 237 | 239 |
| D19S401 | 353 | 353 | 345 | 341 | 353 | 345 | 353 | 341 | 345 | 349 | 353 | 349 | 345 | 349 | 345 | 345 | 341 | 345 | 353 | 349 |
| D19S568 | 256 | 246 | 256 | 268 | 256 | 256 | 256 | 268 | 246 | 246 | 256 | 246 | 246 | 246 | 246 | 246 | 246 | 246 | 256 | 246 |
| D19S434 | 269 | 273 | 273 | 269 | 269 | 273 | 269 | 269 | 270 | 278 | 270 | 278 | 270 | 278 | 270 | 270 | 270 | 270 | 270 | 278 |
| D19S1036 | 208 | 208 | 208 | 204 | 208 | 208 | 208 | 204 | 208 | 204 | 204 | 204 | 208 | 204 | 208 | 208 | 208 | 208 | 204 | 204 |
| Centromere | | | | | | | | | | | | | | | | | | | | |
| D19S419 | 165 | 167 | 167 | 163 | 165 | 167 | 165 | 163 | 165 | 165 | 165 | 165 | 165 | 165 | 165 | 165 | 165 | 165 | 165 | 165 |
| D19S931 | 159 | 161 | 157 | 157 | 159 | 157 | 159 | 157 | 156 | 160 | 164 | 160 | 156 | 160 | 156 | 156 | 156 | 156 | 164 | 160 |
| D19S870 | 256 | 251 | 256 | 251 | 256 | 256 | 256 | 251 | 251 | 256 | 251 | 256 | 251 | 256 | 251 | 251 | 251 | 251 | 251 | 256 |
| D19S222 | 231 | 231 | 231 | 231 | 231 | 231 | 231 | 231 | 239 | 237 | 237 | 237 | 239 | 237 | 239 | 239 | 239 | 239 | 237 | 237 |
| D19S920 | 213 | 213 | 209 | 209 | 213 | 209 | 213 | 209 | 209 | 213 | 209 | 213 | 209 | 213 | 209 | 209 | 209 | 209 | 209 | 213 |
| D19S932 | 141 | 129 | 137 | 135 | 141 | 137 | 141 | 135 | 129 | 135 | 129 | 135 | 129 | 135 | 129 | 129 | 129 | 129 | 129 | 135 |
| D19S875 | 91 | 111 | 105 | 103 | 91 | 105 | 91 | 103 | 107 | 109 | 91 | 109 | 107 | 109 | 107 | 107 | 107 | 107 | 91 | 109 |
| D19S919 | 213 | 209 | 209 | 209 | 213 | 209 | 213 | 209 | 209 | 209 | 211 | 209 | 207 | 209 | 207 | 209 | 207 | 209 | 211 | 209 |
| D19S433 | 196 | 196 | 200 | 215 | 196 | 200 | 196 | 215 | 213 | 203 | 199 | 203 | 213 | 203 | 213 | 213 | 213 | 213 | 199 | 203 |

(*continued*)

**Table 1 (continued)**

| Marker[a] | Genotype[b] in Pedigree | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | X-1 | | X-2 | | XI-1 | | XI-2 | | III-2 | | IV-1 | | IV-2 | | IV-3 | | IV-4 | | IV-5 | |
| D19S405 | *113* | 115 | *107* | 115 | *113* | 107 | *113* | 115 | 113 | 113 | 111 | 113 | 113 | 113 | 113 | 113 | 113 | 113 | 111 | 113 |
| D19S882 | *279* | 277 | *279* | 267 | *279* | 279 | *279* | 267 | 279 | 279 | 267 | 279 | 279 | 279 | 279 | 279 | 279 | 279 | 267 | 279 |
| D19S414 | *181* | 167 | *167* | 167 | *181* | 167 | *181* | 167 | 186 | 184 | 182 | 184 | 186 | 184 | 186 | 186 | 186 | 186 | 182 | 184 |
| D19S225 | *173* | 173 | *167* | 171 | *173* | 167 | *173* | 171 | 169 | 175 | 171 | 175 | 169 | 175 | 169 | 169 | 169 | 169 | 171 | 175 |
| D19S868 | *194* | 198 | *198* | 198 | *194* | 198 | *194* | 198 | 179 | 190 | 190 | 190 | 179 | 190 | 179 | 179 | 179 | 179 | 190 | 190 |
| D19S416 | *168* | 170 | *166* | 172 | *168* | 166 | *168* | 172 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 | 168 |
| D19S425 | *251* | 259 | *271* | 251 | *251* | 271 | *251* | 251 | 251 | 261 | 251 | 261 | 265 | 261 | 265 | 251 | 265 | 251 | 251 | 261 |
| D19S224 | *249* | 233 | *233* | 251 | *249* | 233 | *249* | 251 | 251 | 253 | 251 | 253 | 251 | 253 | 251 | 251 | 251 | 251 | 251 | 253 |
| D19S220 | *283* | 289 | *279* | 285 | *283* | 279 | *283* | 285 | 289 | 279 | 281 | 279 | 279 | 279 | 279 | 289 | 279 | 289 | 281 | 279 |
| D19S420 | *110* | 90 | *100* | 106 | *110* | 100 | *110* | 106 | 106 | 98 | 110 | 98 | 94 | 98 | 94 | 106 | 94 | 106 | 110 | 98 |
| D19S902 | *259* | 244 | *250* | 240 | *259* | 250 | *259* | 240 | 249 | 249 | 239 | 249 | 239 | 249 | 239 | 249 | 239 | 249 | 247 | 249 |
| D19S571 | *287* | 287 | *287* | 313 | *287* | 287 | *287* | 313 | 310 | 310 | 310 | 310 | 310 | 310 | 310 | 310 | 310 | 310 | 310 | 310 |
| D19S418 | *96* | 92 | *92* | 86 | *96* | 92 | *96* | 92 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 |
| D19S877 | *250* | 244 | *240* | 242 | *250* | 240 | *250* | 240 | 261 | 261 | 240 | 261 | 261 | 261 | 240 | 261 | 240 | 261 | 240 | 261 |
| D19S254 | 140 | 132 | *112* | 112 | 132 | 112 | 140 | 112 | 112 | 132 | 112 | 132 | 128 | 132 | 128 | 132 | 112 | 112 | 112 | 112 |
| D19S210 | 177 | 185 | *177* | 179 | 177 | 177 | 177 | 177 | 177 | 175 | 175 | 175 | 177 | 175 | 175 | 177 | 177 | 177 | 177 | 175 |
| D19S890 | 278 | 280 | *282* | 274 | 280 | 282 | 278 | 274 | 280 | 280 | 276 | 280 | 288 | 280 | 288 | 280 | 276 | 280 | 276 | 280 |

[a] Markers are ordered according to NCBI Map Viewer, build 30 (see the Entrez Genome Web site). ABI screening kit markers are underlined; the markers beginning with "M" were established in our laboratory (for details, see table A).

[b] Regions of homozygosity are boxed. Boldface italic numerals refer to the shared chromosomal segments in the Norwegian brothers; roman numerals refer to the pedigrees in figure 1.

the statistical significance of the observed homozygosity. For the Israeli family, the maximum multipoint LOD score within the shared segment was 2.47. Unfortunately, the complexity of the Norwegian pedigree imposed computational constraints, limiting the analysis to three markers at a time. Within the Norwegian family, we obtained a maximum four-point LOD score of 1.75, on the basis of markers D19S895, D19S566, and D19S603. In the absence of ancestral genotypes, this is an underestimate of the true LOD score (see the "Discussion" section). Hence, we used the method of Durham and Feingold (1997) to directly estimate the probability that, in the Norwegian pedigree, we would find such an identical-by-descent (IBD) segment by chance (i.e., without linkage to the disease locus). We estimate this genomewide probability as $p < 0.02$. This value is statistically significant by itself, and the LOD score of 4.22 for the two families combined further supports the significance of the finding.

### *Identification and DNA Sequencing of Candidate Genes*

Chromosome 19 is unusually gene rich. More than 50 confirmed and hypothetical genes reside within this 1.4-Mb candidate region (NCBI Map Viewer, build 30 [see the Entrez Genome Web site]). No detrimental mutations were detected in the coding sequences of the first 24 sequenced genes (table B).

Eventually, DNA sequencing of the cytokine receptor–like factor 1 gene (*CRLF1*) identified homozygosity for a 2-bp deletion (c.844_845delGT) in exon 5 of the *CRLF1* gene in the Norwegian brothers (fig. 2*A*). Such a frameshift mutation will result in a nonfunctional gene product. In the Israeli sisters, homozygosity for two sequence variants was demonstrated, in codons 81 (CGC→CAC) and 374 (CTC→CGC) (figs. 2*B* and 2C). Each substitution is predicted to produce amino acid an change, R81H and L374R, respectively. Neither the deletion nor the substitutions were identified among 200 Norwegian and 50 Israeli control individuals, supporting the assumption that these mutations are causally related to the disorder.

To investigate whether sequence variants in the *CRLF1* gene could be commonly encountered in the population, we sequenced the nine exons of the *CRLF1* gene in 10 unaffected Norwegian blood donors. No variants were found.

We also sequenced the coding sequence of the functionally related *CNTF* gene in all four patients, to investigate whether a variant in this gene could influence the CISS phenotype. A predicted serine→glycine variant in CNTF codon 208 (i.e., heterozygous S208G) was identified in both Norwegian brothers. This sequence variant represents a common polymorphism in the Norwegian population (allele frequency 0.27). No sequence variants were identified in the Israeli sisters.

## Discussion

Our study demonstrated a 1.4-Mb candidate region of homozygosity (IBD) in the Norwegian patients. Be-

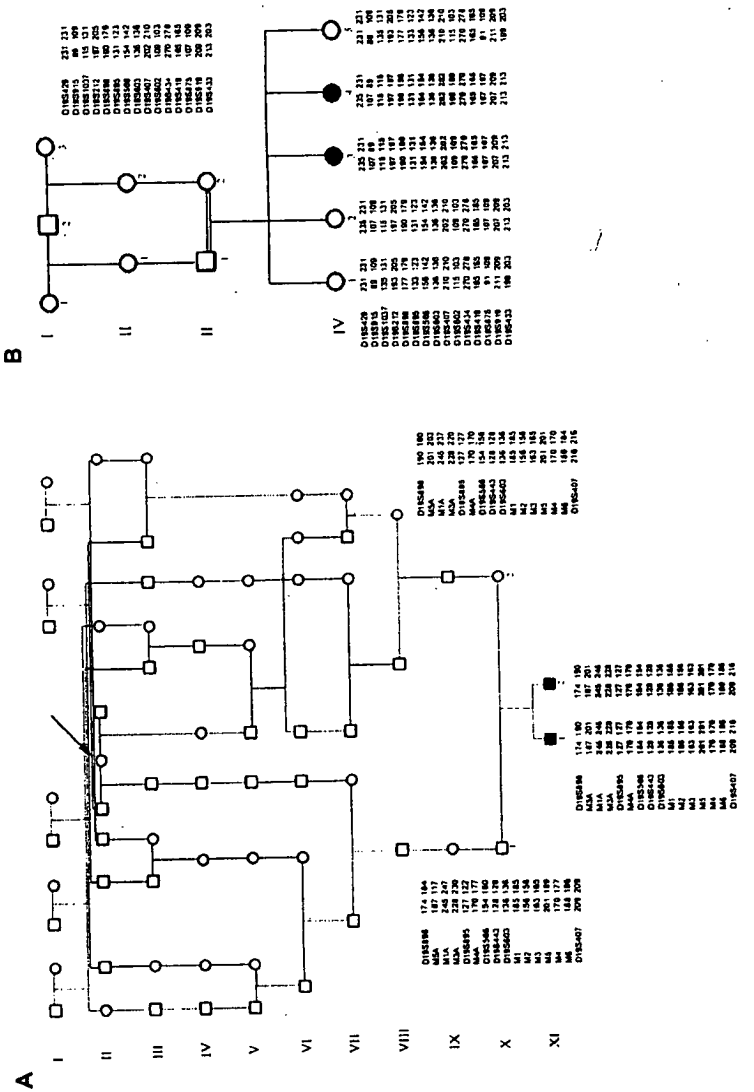


**Figure 1** Pedigree of Norwegian (*A*) and Israeli (*B*) families. Blackened symbols indicate patients with CISS. In the Norwegian family, the first common ancestor was found nine generations back (*arrow*). Observed marker homozygosity is shown in boldface.

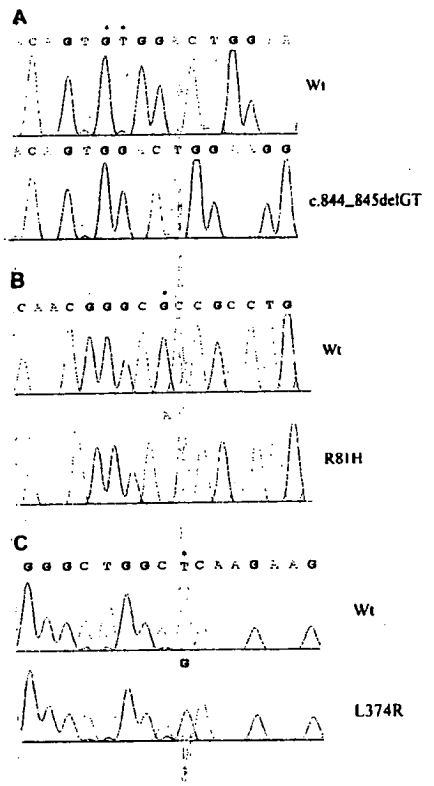


**Figure 2** Mutation analysis of *CRLF1* in Norwegian and Israeli families. Wt = wild type. *A*, DNA sequence of *CRLF1* exon 5, showing the 2-bp deletion (c.844_845delGT). *B*, DNA sequence of *CRLF1* exon 2, showing the A→G substitution in the second position of codon 81, predicting a change from arginine to histidine (R81H). *C*, DNA sequence of *CRLF1* exon 7, showing the T→G substitution in the second position of codon 374, substituting arginine for leucine (L374R). The sites of mutational changes are indicated by asterisks (*). (Numbering of *CRLF1* cDNA here is based on GenBank [accession number NM_004750.2].)

cause of computational constraints, we were not able to calculate an exact multipoint LOD score for the Norwegian pedigree. In the absence of ancestral genotypes, the probability that a shared segment is inherited IBD from a common ancestor increases with the number of informative markers contained in the segment; that is, a shared segment containing only three markers has a significant probability of being simply identical by state, whereas a segment containing a large number of shared markers is much more likely to be IBD from a common ancestor. Thus, the maximum four-point LOD score of 4.22 for the two families combined, on the basis of only three markers from within an interval containing 13 shared markers, is an underestimate of the true LOD score. As an alternative approach, we adapted the method of Durham and Feingold (1997) to estimate the probability that the homozygosity observed in the Norwegian pedigree is a false positive. We estimate this probability as $p < 0.02$. Both of the above approaches provide strong support for the hypothesis that the shared segment on chromosome 19 contains the disease locus.

DNA sequencing of the *CRLF1* gene identified mutations in both the Norwegian brothers and the Israeli sisters. The 2-bp deletion observed in the Norwegian brothers will result in a frameshift encoding a nonfunctional gene product. The substitutions observed in the Israeli sisters are predicted to produce the amino acid changes R81H and L374R. Although the overall phenotype in the Norwegian brothers and the Israeli sisters was similar, the phenotype in the brothers was more severe (e.g., including feeding difficulties, serious kyphoscoliosis, earlier age at onset of the sweating problem, and reduced pain and temperature sensitivity). These differences may be related to different degrees of functional severity of the observed *CRLF1* mutations—namely, a knockout mutation in the Norwegian brothers and the presence of a CRLF1 protein that may have some residual activity in the Israeli sisters. Possibly, other genetic factors (e.g., different sex of the patients) may have also contributed.

CRLF1 is a soluble cytokine receptor with homology to type 1 cytokine receptors (Elson et al. 1998). CRLF1 associates with the cardiotrophin-like cytokine, to form a soluble functional heteromeric ligand, and competes with ciliary neurotrophic factor (CNTF) for the binding to the ciliary neurotrophic factor receptor (CNTFR) complex (Elson et al. 2000). The binding of CRLF1 and CNTF to a common receptor—and their apparent functional similarity—led to the dubbing of CRLF1 as "CNTF II" (Lesser and Lo 2000). CNTF exerts a survival-promoting effect on a variety of neuronal cells. However, the use of CNTF as an experimental treatment of patients with motor-neuron disease did not influence the clinical course of this degenerative disorder (Lambert et al. 2001). Furthermore, a null mutation in the *CNTF* gene occurs as a common variant in the Japanese population and is not associated with any neurological disorder (Takahashi et al. 1994).

To our knowledge, no impaired function of either CRLF1 or any of the other factors constituting the CNTFR complex has so far been implicated in any human disorder. However, some clinical observations



in the Norwegian brothers show similarities to observations made in experimental animals and in cell cultures. In the developing mouse embryo, *CRLF1* is expressed at multiple sites, including skeletal muscle (Elson et al. 1998; Alexander et al. 1999). CNTFR, the receptor for CRLF1, is primarily expressed in the nervous system (Stockli et al. 1991; DeChiara et al. 1995), but expression is also detected in skeletal muscle (Davis et al. 1991). A muscle biopsy performed during back surgery in one of the Norwegian patients showed atrophic skeletal muscle, possibly contributing to the development of his severe kyphoscoliosis. This may indicate that normal CRLF1 exerts an effect not only on neuronal but also on skeletal-muscle development and survival.

In vitro experiments show that CRLF1 can promote the survival of developing embryonic motor neurons (Elson et al. 2000). Mouse models lacking either the *CRLF1*, *CNTFR*, or *CNTF* function have been constructed. A significant reduction in motor-neuron numbers in brain motor nuclei and in the spinal cord has been observed in mice that lack CNTFR (DeChiara et al. 1995), but no structural anomalies have been observed in mice that lack CNTF (Alexander et al. 1999). The metal used for vertebral fixation in the Norwegian boys precludes renewed magnetic-resonance–imaging studies. However, the preoperative images of the cervicothoracic spine show apparently normal dimensions of the spinal cord in both brothers.

Mice lacking the *CRLF1* gene (i.e., $NR6^{-/-}$ mice) were unable to suckle and died of starvation shortly after birth, with their stomachs devoid of milk (Alexander et al. 1999). The newborn mice could open and close their mouths, and no anatomical anomalies were detected on dissection. Alexander et al. (1999) concluded that *CRLF1* was indispensible for suckling, but they were unable to identify the mechanism by which its role was mediated. They have put forth a hypothesis that involves either (*a*) defective recognition or processing of pheromonal signals or (*b*) defective mechanics of suckling itself. Also, newborn mice that lack *CNTFR* are unable to feed; in these mice, impaired jaw movements have been observed.

Both Norwegian patients (but not the Israeli patients) had severe feeding problems as newborns, requiring hospitalization and nasogastric feeding. As children, the brothers continued to show no interest in food. They made many excuses to avoid eating and lagged behind in their growth and development. They both have restricted jaw movements, making dental work difficult, but physical restraint is not a major reason for them not to eat. Interestingly, injections of the related CNTF can cause weight loss in animals and humans, likely to work via a leptinlike pathway on appetite centers in the hypothalamus (Lambert et al. 2001). Thus, it is tempting to speculate that the potentially lethal lack of appetite exerted by a knockout mutation in the *CRLF1* gene may also, in some way, be mediated through malfunction of appetite-regulation centers.

One possibility is that there is normally a physiological competitive binding of CNTF and CRLF1 to their common receptor, CNTFR, at various stages in development (Elson et al. 2000). In the Norwegian patients with a *CRLF1*-knockout mutation, the postulated normal balance between the two ligands competing for the same receptor could be impaired. Since no CRLF1 is produced, their common receptor may be stimulated solely by CNTF, exerting a potentially lethal appetite-depressive effect. Interestingly, leptin has recently been shown to act as a skeletal growth factor, with a direct peripheral effect on the mouse mandibular growth center through a mechanism that is as yet unknown (Maor et al. 2002).

Response to cold is a complex interplay of ion channels in both cold-sensitive and cold-insensitive neurons (Viana et al. 2002). Information on gentle cooling is transmitted by a small subpopulation of sensory nerves, whereas others transmit information on noxious cold and pain. Small changes in the balance of channel expression or in the properties of cold-insensitive neurons may transform cold-insensitive neurons into cold-sensitive fibers (McMemy et al. 2002; Peier et al. 2002; Viana et al. 2002). In all four patients with CISS, the parts of the body surface that sweat profusely at cold temperatures were completely dry under circumstances that normally induce sweating (e.g., hot weather, strenuous exercise, and fever). Thus, the sweat glands in the implicated parts of the body remain under neural control but react inversely to environmental temperatures. The Norwegian patients have impaired peripheral sensitivity to pain and temperature, including the direct exposure to subfreezing cold and steaming heat. Thus, further studies of patients with the *CRLF1*-deficient phenotype may yield information on complex neuronal processing and the interrelationship between various sensory stimuli.

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## Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://www.marshfieldclinic.org/research/genetics/
Cooperative Human Linkage Center, The, http://gai.nci.nih.gov/CHLC/
Digitalarkivet, http://digitalarkivet.uib.no/cgi-win/WebFront.exe?slag=vis&tekst=meldingar
Entrez Genome, http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi? (for NCBI Map Viewer, build 30)
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *CRLF1* cDNA [accession number NM_004750.2])
GenLink, http://www.genlink.wustl.edu/
Genome Database, The, http://www.gdb.org/
LLNL Human Genome Center, http://greengenes.llnl.gov/genome/genome.html
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CISS [MIM 272430])

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# Suckling defect in mice lacking the soluble haemopoietin receptor NR6

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**Cytokines control a variety of cellular responses including proliferation, differentiation, survival and functional activation, via binding to specific receptors expressed on the surface of target cells [1]. The cytokine receptors of the haemopoietin family are defined by the presence of a conserved 200 amino acid extracellular domain known as the haemopoietin domain [2]. We report here the isolation of NR6, a haemopoietin receptor that, like the p40 subunit of interleukin-12 (IL-12) [3] and the EBI3 gene induced by Epstein–Barr virus infection in lymphocytes [4], contains a typical haemopoietin domain but lacks transmembrane and cytoplasmic domains. Although *in situ* hybridisation revealed NR6 expression at multiple sites in the developing embryo, mice lacking NR6 did not display obvious abnormalities and were born in the expected numbers. Neonatal NR6$^{-/-}$ mice failed to suckle, however, and died within 24 hours of birth, suggesting that NR6 is necessary for the recognition or processing of pheromonal signals or for the mechanics of suckling itself. In addition, NR6$^{-/-}$ mice had reduced numbers of haemopoietic progenitor cells, suggesting a potential role in the regulation of primitive haemopoiesis.**

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## Results and discussion

Using an oligonucleotide encoding the conserved WSXWS (single-letter amino acid code) motif present in the extracellular domains of hemopoietin receptors [2], a cDNA denoted NR6 was isolated from murine testis, brain and KUSA cell line [5] cDNA libraries. The majority of clones contained a long open-reading frame (NR6.1) of 1275 nucleotides. The predicted protein sequence was consistent with that of a haemopoietin receptor [2]: a potential signal sequence and immunoglobulin-like domain preceded a haemopoietin domain (HD) containing the expected cysteine pairs and a WSEWS motif, and sequence with loose homology to part of the fibronectin type III repeat was evident at the carboxyl terminus. Independent clones were also isolated with deduced open reading frames (NR6.2 and NR6.3) that contained divergent sequences carboxy-terminal to the HD (Figure 1). Human NR6 cDNAs, all of which were homologues of murine NR6.1, were isolated using low-stringency hybridisation of murine probes to foetal kidney, foetal liver and placental libraries (Figure 1). No hydrophobic sequences typical of a transmembrane domain nor motifs usually required for membrane association via lipid attachment [6] were evident, indicating that NR6 is a soluble member of the haemopoietin receptor family. The primary amino-acid sequences of human and mouse NR6.1 were 98% identical and are identical to the recently described cytokine-like factor-1 (CLF-1) [7]. Although NR6 has sequence similarity to membrane-bound haemopoietin receptors (Figure 1), structurally it appears to be analogous to the two other exclusively soluble members of the haemopoietin receptor family — EBI3, a 34 kDa glycoprotein secreted by B lymphocytes in response to EBV [4], and the p40 component of IL-12 [3].

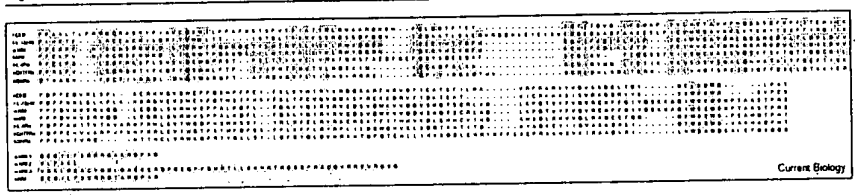
Embryos at 7.5–11.5 days post coitum (dpc) were examined for NR6 expression by whole-mount *in situ* hybridisation with digoxigenin-labelled riboprobes. NR6 expression was first detected at 9.5 dpc in the first branchial arch, the forelimb bud and mesonephric duct (Figure 2a). At 10.5 and 11.5 dpc, intense expression was seen in nasal processes and the maxillary and mandibular components of the first branchial arch. Expression was also seen in the limbs, in the mesenchyme overlying the otic vesicle and in the dermatomyotome (Figure 2b). Expression of NR6 in 12.5, 14.5 and 18.5 dpc embryos was examined by *in situ* hybridisation of radiolabelled probes to tissue sections. At each age, NR6 was expressed in the craniofacial mesenchyme and in tissues derived from the first branchial arch. At 14.5 and 18.5 dpc, NR6 transcripts were also observed in dental papillae, in the tongue and throughout the mesenchyme beneath the oral and nasal epithelia (Figure 2e). Expression was observed in the secretory buds and ducts of the submandibular salivary gland from 14.5 dpc and in the lacrimal glands at 18.5 dpc (Figure 2e). At 12.5 and 14.5 dpc the ectoderm of Rathke's pouch expressed NR6, although transcripts were not detected in the pituitary gland. NR6 was expressed in the mesonephric (Wolffian) duct at 12.5 dpc and in the growing tips of the collecting ducts of the kidney throughout embryogenesis (Figure 2c). Expression was observed in the genital tubercle but was not detected in other reproductive organs (Figure 2c). NR6 transcripts were detected in the lung buds at 12.5 dpc and in the bronchi, but not in lung parenchyma, at 14.5 and 18.5 dpc (Figure 2c). From 12.5 dpc, NR6 expression was observed in all precartilaginous membranous blastema. At later times, expression was prominent in tissues adjacent to forming cartilage, such as the intermediate digits of the hindlimb (Figure 2c). NR6 transcripts were not detected in the developing brain before 17.5 dpc. At this time, expression was observed in the nuclear zone of the neopallial cortex and in the hippocampus (Figure 2c). Rare NR6-positive cells were also observed in the midbrain. By birth, expression of NR6 in the brain was no longer detectable.

To examine the biological role of NR6 *in vivo*, a targeting vector in which the NR6 immunoglobulin-like and haemopoietin domains were replaced with a G418-resistance cassette (Figure 3a) was used for homologous recombination in embryonic stem (ES) cells to generate mice in which the NR6 gene had been functionally deleted. A number of mice in litters born of NR6$^{+/-}$ parents died within 24 hours of birth. Genotyping revealed that these mice were homozygous for the targeted NR6 allele, whereas their healthy littermates were heterozygotes or wild type (Figure 3b). As anticipated, NR6 transcripts were detected in northern blot analysis of RNA from NR6$^{+/-}$ or wild-type mice, but were absent in samples from homozygous mutants (Figure 3c). Although NR6$^{-/-}$ mice were born in numbers expected from normal Mendelian segregation of alleles, no NR6$^{-/-}$ mice survived beyond 24 hours after birth. Thus loss of NR6 does not compromise embryonic survival but is lethal during the first day of life.

Extensive histological comparison of serial sections from neonatal NR6$^{-/-}$ mice and wild-type littermates revealed

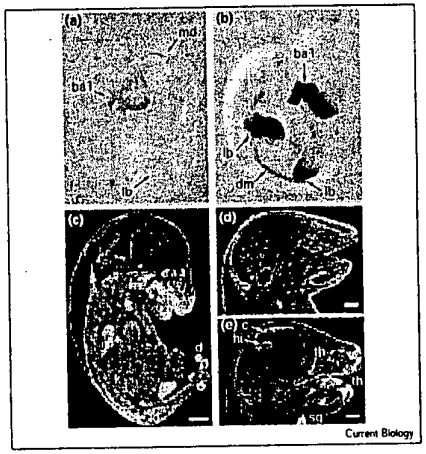


**Figure 1**



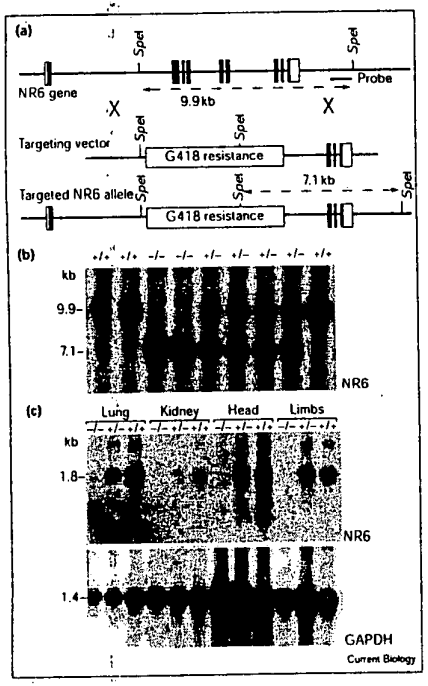

NR6 is a member of the haemopoietin receptor family. Alignment of the amino-acid sequence of murine (mNR6) and human (hNR6) NR6 with those of human Epstein–Barr virus-induced protein (hEBI3), the p40 component of human IL-12 (hIL-12p40), and the human receptors for IL-6 (hIL-6Rα), ciliary neurotrophic factor (hCNTFRα) and granulocyte-macrophage colony-stimulating factor (hGMRα). Conserved residues in the SD100A (grey) and SD100B (blue) regions of the haemopoietin domain are shaded, with the characteristic cysteine pairs and WSXWS motifs in bold. The carboxy-terminal sequences that diverge in mNR6.1, mNR6.2 and mNR6.3 are shown in pink together with the hNR6 carboxyl terminus.

**Figure 2**




Expression of NR6 in the mouse embryo. (a,b) Whole-mount *in situ* hybridisation of (a) 9.5 dpc and (b) 11.5 dpc embryos showing NR6 expression in the mesonephric duct (md), limb buds (lb), first branchial arch (ba1), nasal processes and dermatomyotome (dm). (c) Sagittal section of a 14.5 dpc embryo showing NR6 expression in lung (l), kidney (k), genital tubercle (gt), precartilaginous condensations of the digital metacarpals (d), intervertebral discs (id), tongue (t) and facial mesenchyme. (d,e) Serial sagittal sections of the head from an 18.5 dpc embryo, hybridised with (d) sense and (e) antisense probes revealing NR6 expression in the cortex (c) and hippocampus (hi), as well as in facial mesenchyme, developing teeth (th) and salivary gland (sg). Scale bars, 1 mm. Hybridisation of a $^{33}$P-labelled full-length NR6 cDNA probe to whole-mount (70°C) and embryonic paraffin sections (50°C) were performed as described previously [12,13].

Figure 3




Gene targeting of the NR6 locus. (a) Structure of the murine NR6 gene with exons boxed and coding region as filled boxes. The targeting vector and the predicted structure of the targeted allele are shown. (b) Southern blot of *Spe*I-digested genomic DNA from the tails of mice from a cross between heterozygous ($NR6^{+/-}$) parents. The endogenous (9.9 kb) and mutant (7.1 kb) NR6 alleles were detected by the genomic NR6 probe. (c) Northern blot analysis of RNA extracted from the lungs, kidneys, heads and limbs of neonatal $NR6^{-/-}$, wild-type (+/+) and heterozygous (+/–) mice using full-length NR6 cDNA and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes.

no gross structural abnormalities or histopathology. Specific staining of bone and cartilage in cleared embryos [8] collected the day before birth also failed to detect any consistent skeletal abnormalities (data not shown). Closer examination soon after birth revealed that $NR6^{-/-}$ mice failed to suckle effectively and had stomachs devoid of milk (Figure 4). The $NR6^{-/-}$ mice had normal body weights, a normal respiratory rate, were well oxygenated and responded to touch with vocalisation, righting and rooting reflexes. They could open and close their mouths and dissections revealed that the palate, mouth and oesophagus were intact. Suckling is thought to be initiated by pheromonal responses which are processed by neural

Figure 4

Failure to suckle in $NR6^{-/-}$ mice. A litter of living newborn mice from a cross between $NR6^{+/-}$ parents, showing the empty stomachs of three $NR6^{-/-}$ mice (left). Wild-type or $NR6^{+/-}$ mice, four of which are shown at the right, suckle normally.

networks involving the olfactory bulb, and ultimately, the hippocampus [9]. Indeed, in mice lacking the Fyn tyrosine kinase, a failure to suckle has been correlated with abnormalities at these sites [10]. In contrast, in mice lacking the Brn-3a POU domain protein, neuronal loss in the trigeminal ganglion accompanied a failure to suckle, suggesting that sensory defects in the face or mouth may also impair this response [11]. The brains of two newborn $NR6^{-/-}$ mice and two wild-type littermates were serially sectioned in the coronal plane and every fifth section was photographed. Sagittal sections through the brain, face and mouth of several animals were also examined. No abnormalities were observed in the anatomy of the $NR6^{-/-}$ brains, including the cortex and hippocampus, the two sites of NR6 expression, and the olfactory bulb. The complete brain stem was not examined. Similarly, gross structural or histological abnormalities of the face and mouth were not evident. Thus, although our data establish that NR6 is indispensable in the initiation and/or maintenance of suckling in neonatal mice, and reveal NR6 expression in facial and neural sites previously implicated in suckling, we have not been able to identify the mechanism by which its role is mediated. It seems likely, however, that the expression of NR6 in facial tissues and/or the brain is necessary for the recognition or processing of pheromonal signals or for the mechanics of suckling itself.

We also examined whether haemopoiesis was perturbed in $NR6^{-/-}$ mice. The haematocrit number of circulating platelets and number and morphological distribution of white blood cells were normal in $NR6^{-/-}$ mice. The numbers and lineage commitment of haemopoietic progenitor cells in neonatal mice were enumerated in clonogenic cultures. $NR6^{-/-}$ bone marrow and spleen contained 1.5–2.5-fold fewer progenitors capable of responding to the combination of stem-cell factor (SCF), IL-3 and erythropoietin (EPO). A similar reduction in the number of clonogenic cells responsive to macrophage colony-stimulating factor (M-CSF) or SCF alone was also observed (data not shown). The deficiencies did not reflect a reduction in cells



Table 1

**Haemopoietic progenitor cell profile in neonatal NR6-deficient mice.**

| Organ | NR6 genotype | Number of colonies per $2 \times 10^4$ cells | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Blast | G | GM | M | Eo | E | Meg | E/Meg | Mix |
| BM | +/+ | 125 ± 13 | 9 ± 2 | 37 ± 2 | 34 ± 13 | 21 ± 1 | 2 ± 1 | 5 ± 6 | 12 ± 6 | 4 ± 2 | 1 ± 2 |
| | +/– | 102 ± 30 | 6 ± 4 | 36 ± 17 | 23 ± 3 | 11 ± 3 | 2 ± 1 | 6 ± 6 | 9 ± 7 | 8 ± 3 | 1 ± 2 |
| | –/– | 78 ± 23* | 5 ± 3 | 23 ± 3 | 22 ± 10 | 10 ± 3 | 2 ± 2 | 5 ± 1 | 8 ± 2 | 3 ± 2 | 1 ± 1 |
| Spleen | +/+ | 98 ± 6 | 8 ± 3 | 27 ± 1 | 22 ± 4 | 17 ± 2 | 2 ± 0 | 10 ± 1 | 7 ± 5 | 7 ± 1 | 0.7 ± 0.6 |
| | +/– | 66 ± 12* | 3 ± 3 | 18 ± 3 | 17 ± 6 | 16 ± 5 | 1 ± 2 | 4 ± 3 | 3 ± 1 | 3 ± 5 | 0.7 ± 0.6 |
| | –/– | 42 ± 9* | 4 ± 3 | 12 ± 4 | 9 ± 3 | 6 ± 2 | 0.3 ± 0.6 | 4 ± 1 | 3 ± 1 | 4 ± 3 | 0.5 ± 0.6 |
| Liver | +/+ | 63 ± 9 | 6 ± 2 | 18 ± 5 | 14 ± 5 | 16 ± 9 | 0 | 3 ± 1 | 3 ± 4 | 2 ± 1 | 0.7 ± 0.6 |
| | +/– | 74 ± 13 | 10 ± 2 | 18 ± 3 | 15 ± 7 | 18 ± 3 | 0.7 ± 0.6 | 7 ± 5 | 2 ± 1 | 3 ± 2 | 0.7 ± 0.6 |
| | –/– | 62 ± 13 | 5 ± 2 | 19 ± 8 | 15 ± 2 | 10 ± 4 | 0 | 6 ± 3 | 5 ± 4 | 2 ± 1 | 0.5 ± 0.6 |

Mean ± standard deviations of colony numbers from neonatal $NR6^{+/+}$ $NR6^{+/-}$ or $NR6^{-/-}$ bone marrow, spleen or liver. Replicate cultures ($2 \times 10^4$ cells) from each mouse were stimulated with 10 ng/ml IL-3, 100 ng/ml SCF and 4 U/ml EPO and incubated for 7 days in a humidified atmosphere of 5% $CO_2$ in air. Statistical comparisons were performed using Student's *t*-test on total colony data from $NR6^{+/-}$ or $NR6^{-/-}$ mice versus $NR6^{+/+}$. *$p < 0.05$, $n = 3$ or 4 mice of each genotype. BM, bone marrow; G, granulocyte; GM, granulocyte-macrophage; M, macrophage; Eo, eosinophil; E, erythroid; Meg, megakaryocyte; E/Meg, mixed erythroid/megakaryocyte; Mix, colonies containing cells of three or more lineages.

committed to any particular lineage; fewer colonies of all types monitored were evident (Table 1). The numbers and lineage commitment of haemopoietic progenitor cells were normal in the livers of neonatal $NR6^{-/-}$ mice (Table 1), and in similar analyses, no disturbances in foetal liver progenitor numbers or lineage commitment were evident at day 13 of gestation (data not shown). Although it is possible that the debilitating effects of the failure to suckle in these mice causes marrow and spleen progenitor cell numbers to quickly decrease after birth, the intact response of liver progenitors from the same animals suggests that this is not the case. Rather, NR6 may be required for progenitor cell production in spleen and bone marrow late in gestation. In support of a potential role as a haemopoietic regulator, NR6 is expressed by a number of stromal cell lines known to support haemopoiesis (data not shown).

Thus, NR6 is indispensable for suckling, with lethal consequences in neonatal mice lacking this protein. It is also required for appropriate production of haemopoietic progenitor cells in the bone marrow and spleen. Further studies are required to define this soluble haemopoietin receptor biochemically and to further explore its contributions to these complex biological processes.

*Supplementary material*

Additional methodological details are published with this paper on the internet.

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# Supplementary material

# Suckling defect in mice lacking the soluble haemopoietin receptor NR6

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## Supplementary materials and methods

### *cDNA cloning*

Mouse testis, brain (Stratagene) and KUSA cell cDNA libraries were screened with an oligonucleotide complementary to the sequence encoding the WSXWS motif as previously described [S1]. A total of 18 cDNA clones were obtained which appeared to encode a novel member of the haemopoietin receptor family, NR6. Murine cDNA inserts were then used as hybridisation probes under low stringency conditions to isolate human NR6 cDNAs from foetal liver, foetal kidney and placental libraries (Stratagene) using standard techniques.

### *RNA expression analyses*

*In situ* hybridisation analyses were performed on tissues that were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned. Hybridisation to whole-mount and embryonic sections were performed as described previously [S2,S3], except that embryos and sections were treated with 20 mg/ml proteinase K (Boehringer Mannheim). The full length NR6 cDNA was used as probe. Whole mount *in situ* hybridisation was performed at 70°C and *in situ* hybridisation to tissue sections was performed at 50°C.

### *Generation of NR6-deficient mice*

To construct the NR6 targeting vector, 4.1 kb of murine genomic NR6 DNA containing exons 2 through to 6 was deleted and replaced with a G418-resistance cassette, leaving 5′ and 3′ arms of NR6 homology of 2.9 and 4.5 kb, respectively. The targetting vector was linearised and electroporated into W9.5 embryonic stem cells. Colonies of cells resistant to 175 µg/ml G418 were picked and expanded after 8 days in selection medium. Clones in which the targeting vector had recombined with the endogenous NR6 gene were identified by hybridising *Spe*I-digested genomic DNA with a 0.6 kb *Xho*I–*Sca*I genomic NR6 fragment, which distinguished between the endogenous (9.9 kb) and targeted (7.1 kb) NR6 loci. Homologous recombination at the NR6 locus was observed in 19 of 158 clones analysed (12%). Two targeted ES cell clones were injected into C57Bl/6 blastocysts to generate chimeric mice. Male chimaeras were mated with C57Bl/6 females to yield NR6 heterozygotes, which were subsequently interbred to produce wild-type ($NR6^{+/+}$), heterozygous ($NR6^{+/-}$) and mutant ($NR6^{-/-}$) offspring. The genotypes of offspring were determined by Southern blot analysis of genomic DNA extracted from tail biopsies. DNA extraction, digestion with restriction endonucleases and processing of Southern blots were performed as described [S4].

### *Histological and haematological analyses*

Neonatal $NR6^{-/-}$ mice were compared histologically with normal littermates using whole animal serial sagittal sections following fixation in Bouin's fixative and staining with haematoxylin and eosin (H&E). Morphological studies of the brain were conducted on H&E-stained serial coronal sections of tissues fixed in 4% paraformaldehyde. Sagittal sections of the brain, face and mouth of several animals were also examined. In this experiment, although not in other anatomical studies, the histology of the brains of the newborn NR6 null mice was slightly inferior to that of the control, possibly due to failure of $NR6^{-/-}$ mice to feed causing progressive development of metabolic abnormalities. The clonal culture of haemopoietic progenitor cells was performed in 1 ml cultures of $10^4$ (foetal liver) or $2 \times 10^4$ (neonatal bone marrow, spleen or liver) cells in 0.3% agar in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS), 10 ng/ml murine IL-3, 100 ng/ml murine (stem cell factor) SCF and 4 U/ml human EPO. Parallel cultures were stimulated using 100 ng/ml SCF or 10 ng/ml M-CSF. Agar cultures were fixed and sequentially stained for acetylcholinesterase, Luxol fast blue and haematoxylin, and the composition of each colony was determined at 100–400-fold magnifications as previously described [S5].

## Supplementary references

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